Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for Titration of Porcine Rotavirus in Modified-live Vaccines

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Supplemental Assay Method for the Titration of Porcine Rotavirus in Modified-live Vaccines

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1. Introduction

1.1 Background

This Supplemental Assay Method (SAM) is an *in vitro* assay method which utilizes cytopathic effect (CPE) in a cell culture system or an indirect fluorescent antibody (IFA) technique for determining the Group A porcine rotavirus (PROTA) content of modified-live vaccines.

Note: For this SAM, the dilution terminology of 1:10, 1:20, etc., specifies 1 part plus 9 parts (liquid), 1 part plus 19 parts, etc.

1.2 Keywords

Porcine rotavirus, PROTA, CPE, IFA, potency test, titration, *in vitro*

2. Materials

2.1 Equipment/instrumentation

2.1.1 $36^0 \pm 2^0$C, 5% ± 1% CO_2, high-humidity incubator[1] meeting the requirements in the current version of GDOCSOP0004

2.1.2 Water bath[2]

2.1.3 Microscope, inverted light[3]

2.1.4 Microscope, ultraviolet (UV) light[4]

2.1.5 Vortex mixer[5]

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[1] Model 3158, Forma Scientific, Inc., Box 649, Marietta, OH 45750-0649 or equivalent
[2] Cat. No. 15-461-10, Fisher Scientific, Inc., 319 West Ontario, Chicago, IL 60610 or equivalent
[3] Model CK, Olympus America, Inc., 2 Corporate Center Dr., Melville, NY 11747-3157 or equivalent
[4] Model BH2, Olympus America, Inc. or equivalent
[5] Vortex-2 Genie, Model G-560, Scientific Industries, Inc., 70 Orville Dr., Bohemia, NY 11716 or equivalent

2.1.6 Micropipettors: 200 µl and 1000 µl single channel;[6] 300 µl x 12 channel[7]

2.1.7 Self-refilling repetitive syringe, 2 ml[8]

2.2 Reagents/supplies

2.2.1 PROTA Reference Viruses[9]

2.2.1.1 Serotype 4 (Gottfried)

2.2.1.2 Serotype 5 (OSU strain)

2.2.2 Rhesus monkey kidney cells (MA-104)[10] free of extraneous agents as tested by the Code of Federal Regulations, Title 9 (9 CFR)

2.2.3 Minimum essential medium (MEM)

2.2.3.1 9.61 g MEM[11]

2.2.3.2 2.2 g sodium bicarbonate ($NaHCO_3$)[12]

2.2.3.3 Q.S. to 1000 ml with deionized water (DW); adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).[13]

2.2.3.4 Sterilize through a 0.22-µm filter.[14]

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[6] Pipetman®, Rainin Instrument Co., Mack Rd., Box 4026, Woburn, MA 01888 or equivalent
[7] Finnpipettes®, Cat. No. NX204662D, A. Daigger Company, Inc., 199 Carpenter Ave., Wheeling, IL 60090 or equivalent
[8] Wheaton® 13-689-50C, Fisher Scientific Corp., 2000 Park Ln., Pittsburgh, PA 15275 or equivalent
[9] Reference quantities are available on request from the Center for Veterinary Biologics-Laboratory (CVB-L), P.O. Box 844, Ames, IA 50010
[10] Available on request from the CVB-L or equivalent
[11] MEM with Earle's salts without sodium bicarbonate, Cat. No. 410-1500EF, Life Technologies, Inc., 8400 Helgerman Ct., Gaithersburg, MD 20884 or equivalent
[12] Cat. No. S 5761, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178 or equivalent
[13] Cat. No. 9535-01, J.T. Baker, Inc., 222 Red School Ln., Phillipsburg, NJ 08865 or equivalent
[14] Cat. No. 12122, Gelman Sciences, 600 S. Wagner Rd., Ann Arbor, MI 48106 or equivalent

2.2.3.5 Aseptically add:

1. 10 ml L-glutamine[15]

2. 2.5 µg/ml amphotericin B[16]

3. 100 units/ml penicillin[17]

4. 50 µg/ml gentamicin sulfate[18]

5. 100 µg/ml streptomycin[19]

2.2.3.6 Store at 4⁰ ± 2⁰C.

2.2.4 Growth Medium

2.2.4.1 930 ml of MEM

2.2.4.2 Aseptically add 70 ml of gamma-irradiated fetal bovine serum (FBS).

2.2.4.3 Store at 4⁰ ± 2⁰C.

2.2.5 Diluent Medium

2.2.5.1 100 ml MEM

2.2.5.2 83.3 µl pancreatin[20]

2.2.5.3 Store at 4⁰ ± 2⁰C.

2.2.6 Anti-PROTA monoclonal antibody (MAb)[10]

2.2.6.1 MAb against Serotype 4 (Gottfried)

2.2.6.2 MAb against Serotype 5 (OSU strain)

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[15] L-glutamine-200 mM (100X), liquid, Cat. No. 320-503PE, Life Technologies, Inc. or equivalent
[16] Cat. No. A 2411, Sigma Chemical Co. or equivalent
[17] Cat. No. 0049-0530-28, Schering Laboratories, 2000-T Galloping Hill Rd., Kenilworth, NJ 07033 or equivalent.
[18] Cat. No. 0061-0464-04, Schering Laboratories or equivalent
[19] Cat. No. S-9137, Sigma Chemical Co. or equivalent
[20] Pancreatin 4XNF (10X), Cat. No. 610-5720AG, Life Technologies, Inc. or equivalent

2.2.7 Rabbit anti-mouse fluorescein isothiocyanate-labeled conjugate[21] (Rabbit Anti-mouse Conjugate)

2.2.8 Anti-PROTA serum[9] against OSU and Gottfried strains

2.2.9 0.01 M Phosphate buffered saline (PBS)

2.2.9.1 1.19 g sodium phosphate, dibasic, anhydrous (Na_2HPO_4)[22]

2.2.9.2 0.22 g sodium phosphate, monobasic, monohydrate ($NaH_2PO_4 \cdot H_2O$)[23]

2.2.9.3 8.5 g sodium chloride (NaCl)[24]

2.2.9.4 Q.S. to 1000 ml with DW.

2.2.9.5 Adjust pH to 7.2-7.6 with 0.1 N sodium hydroxide (NaOH)[25] or 2.0 N HCl.

2.2.9.6 Sterilize by autoclaving at 15 psi, $121^0 \pm 2^0$C for 35 ± 5 min.

2.2.9.7 Store at $4^0 \pm 2^0$C.

2.2.10 80% Acetone

2.2.10.1 80 ml acetone[27]

2.2.10.2 20 ml DW

2.2.10.3 Store at room temperature (RT) ($23^0 \pm 2^0$C).

2.2.11 Cell culture plates, 96 well[26]

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[21] Cat. No. 04-6111, Zymed Laboratories, Inc., 458 Carlton Ct., So. San Francisco, CA 94080 or equivalent
[22] Cat. No. S 0876, Sigma Chemical Co. or equivalent
[23] Cat. No. S 9638, Sigma Chemical Co. or equivalent
[24] Cat. No. S 9625, Sigma Chemical Co. or equivalent
[25] Cat. No. 925-30, Sigma Chemical Co. or equivalent
[26] Costar® 3596, Costar Corp., 1 Alewife Center, Cambridge, MA 02140 or equivalent

2.2.12 Polystyrene tubes, 12 x 75 mm[27]

2.2.13 Graduated cylinders, 25 ml, 50 ml, 100 ml, and 250 ml[28], sterile

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel must have training in the immunological basis of cell culture techniques, the principles of IFA, and aseptic technique.

3.2 Preparation of equipment/instrumentation

On the day of test initiation, set a water bath at $36^0 \pm 2^0$C.

3.3 Preparation of reagents/control procedures

3.3.1 MA-104 Plates. Two days prior to test initiation, seed 96-well cell culture plates with MA-104 cells, in Growth Medium, at a cell count that will produce a monolayer after 48 ± 8 hr of incubation at $36^0 \pm 2^0$C in a CO_2 incubator. These become the MA-104 Plates. One test serial or 1 of the 2 PROTA Reference Viruses can be tested on each MA-104 Plate. Growth Medium is changed if excess acidity of the medium is observed or cells are not confluent after incubation.

3.3.2 PROTA Reference Virus-Specific Antiserum Mixture

3.3.2.1 On the day of test initiation, rapidly thaw a vial of each PROTA Reference Virus in a $36^0 \pm 2^0$C water bath.

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[27] Falcon® 2058, Becton Dickinson Labware, 2 Bridgewater Ln., Lincoln Park, NJ 07035 or equivalent

[28] Cat. No. P34546-02, P34546-03, P34546-04, and P34546-05 respectively, Cole-Parmer Instrument Co., 625 Bunker Court, Vernon Hills, IL 60061-9872 or equivalent

3.3.2.2 Pipette 2.0 ml of Dilution Medium into 2, 12 x 75-mm polystyrene tubes using a repetitive syringe. Label each tube with the appropriate reference virus.

3.3.2.3 Transfer 500 µl of each Reference Virus to the corresponding labeled tube; mix by vortexing. Discard pipette tip. This is a 1:5 dilution of the Reference Virus.

3.3.2.4 Transfer 1.0 ml of each 1:5 Reference Virus to 2, 12 x 75-mm polystyrene tubes labeled 10^{-1} for each Reference Virus (total of 4 tubes).

3.3.2.5 Pipette 1.0 ml/tube of a specific Anti-PROTA serum to separate 10^{-1}-labeled tubes of each Reference Virus. This becomes the Reference Virus-Specific Antiserum Mixture. Repeat for the other specific Anti-PROTA serum (see **Section 9.1**).

3.3.2.6 Mix with the vortex mixer; incubate for 120 ± 10 min at RT.

3.3.2.7 Pipette 1.8 ml/tube of Diluent Medium into 2 sets of 12 x 75-mm polystyrene tubes labeled 10^{-2} through 10^{-8} for each Reference Virus-Specific Antiserum Mixture.

3.3.2.8 Prepare serial tenfold dilutions of each PROTA Reference Virus-Specific Antiserum Mixture. Transfer 200 µl from each Reference Virus-Specific Antiserum Mixture tube to the corresponding labeled 10^{-2} tube. Mix with the vortex mixer. Discard pipette tip.

3.3.2.9 Repeat **Section 3.3.2.8** for the remaining tubes, transferring 200 µl sequentially from the previous dilution to the next dilution, until the dilution sequence is completed.

3.3.3 Working Anti-PROTA MAb. On the day of MA-104 plate examination, if an IFA test is to be conducted, dilute the appropriate Anti-PROTA MAb in PBS, according

to the CVB-L supplied Reference and Reagent Sheet or as determined for that specific MAb.

3.3.4 Working Rabbit Anti-mouse Conjugate. On the day of MA-104 plate examination, if an IFA test is to be conducted, dilute the Rabbit Anti-mouse Conjugate in PBS, according to the manufacturer's recommendations.

3.4 Preparation of the sample

3.4.1 Conduct the initial test of a Test Serial with a single vial (a single sample from 1 vial). On the day of test initiation, remove the seal and stopper from both the Test Serial bottle and the bottle containing the accompanying diluent. Measure the diluent into a sterile graduated cylinder according to the number of doses indicated on the manufacturer's instructions (e.g., for a 50-dose container of 2 ml per dose, reconstitute with 100 ml of diluent), and aseptically pour the diluent into the lyophilized bottle of vaccine. Mix by vortexing.

3.4.2 Neutralize Test Serials containing multiple PROTA similar to the methods in **Sections 3.3.2.2 through 3.3.2.6** (see **Section 9.1**).

3.4.3 Make serial tenfold dilutions of the Test Serial similar to the methods in **Sections 3.3.2.7 through 3.3.2.9**.

4. Performance of the test

4.1 On the day of test initiation, decant Growth Medium from the MA-104 Plates.

4.2 Add 200 µl/well Diluent Medium to the MA-104 Plates. Decant the Diluent Medium.

4.3 Again, add 200 µl/well Diluent Medium to the MA-104 Plates and incubate for 60 ± 10 min at $36^0 \pm 2^0$C in a CO_2 incubator. Decant the Diluent Medium.

4.4 Inoculate 200 µl/well of each dilution of the Test Serial and each Reference Virus-Specific Antiserum Mixture into a minimum of 5 wells/dilution of an MA-104 Plate.

4.5 Add 200 µl/well Diluent Medium to a minimum of 5 wells on each MA-104 plate, to serve as uninoculated cell controls.

4.6 Incubate the MA-104 Plates for 120 ± 12 hr postinoculation (HPI) at $36^0 \pm 2^0$C in a CO_2 incubator.

4.7 CPE counting is the primary method of determining the $\log_{10}$ 50% tissue culture infective dose ($TCID_{50}$).

4.7.1 120 ± 12 HPI, examine the wells with an inverted light microscope. The CPE of PROTA is visible as cell death in the cell monolayer.

4.7.2 Record the number of wells/dilution showing any characteristic PROTA CPE for each Test Serial and Reference Virus-Specific Antiserum Mixture.

4.7.3 Calculate the $TCID_{50}$ of the Test Serial and each Reference Virus-Specific Antiserum Mixture using the method of Spearman-Kärber as modified by Finney.

Example:
10^0 dilution of the Test Serial = 5 of 5 wells CPE Pos
10^{-1} dilution of the Test Serial = 5 of 5 wells CPE Pos
10^{-2} dilution of the Test Serial = 3 of 5 wells CPE Pos
10^{-3} dilution of the Test Serial = 0 of 5 wells CPE Pos

Titer = (X - d/2 + [d * S]) where:
X = Log_{10} of lowest dilution (=0)
d = Log_{10} of dilution factor (=1)
S = Sum of proportion of CPE positive (13/5=2.6)

Titer = (0 - d/2 + [1 * 1.6])= 2.1

antilog of 2.1 = 125.9

Titer is 126 $TCID_{50}$.

4.8 Certain strains of PROTA may not exhibit pronounced CPE. An IFA may be conducted to determine the titer:

4.8.1 Decant Growth Media from the MA-104 Plates.

4.8.2 Rinse the MA-104 Plates with PBS and incubate at RT for 5 ± 2 min. Decant the PBS.

4.8.3 Fill the wells with 80% Acetone and incubate at RT for 15 ± 5 min.

4.8.4 Decant the 80% Acetone from the MA-104 Plates and air dry at RT.

4.8.5 Pipette 35 µl of the Working Anti-PROTA MAb into all wells and incubate for 45 ± 15 min at RT.

4.8.6 Fill the wells completely with PBS and incubate at RT for 5 ± 2 min. Decant the PBS.

4.8.7 Repeat for a total of 2 washes.

4.8.8 Gently tap the MA-104 Plates onto paper towels to remove excess liquid.

4.8.9 Pipette 35 µl of the Working Rabbit Anti-mouse Conjugate into all wells and incubate for 40 ± 10 min at RT.

4.8.10 Repeat **Sections 4.9.6 through 4.9.8.**

4.8.11 Dip the plate in DW; decant. Allow to air dry or dry at 36^0 ± 2^0C.

4.8.12 Examine the MA-104 Plates with a UV-light microscope at 100 to 200 X magnification.

4.8.13 A well is considered positive if typical cytoplasmic, apple-green fluorescence is observed.

4.8.14 Record and calculate as in **Sections 4.7.2** and **4.7.3.**

5. Interpretation of the test results

5.1 The test is not a valid test if CPE, fluorescence, or bacterial/fungal contamination is observed in any of the uninoculated control wells.

5.2 An Anti-PROTA serum must neutralize the corresponding homologous Reference Virus.

5.3 The calculated titer of each Reference Virus must fall within plus or minus 2 standard deviations (± 2 SD) of its mean titer, as established from a minimum of 10 previously determined titers.

5.4 If the validity requirements are not met, then the assay is considered a **NO TEST** and can be retested without prejudice.

5.5 If the validity requirements are met and the titer of the Test Serial is greater than or equal to the titer contained in the Animal and Plant Health Inspection Service (APHIS) filed Outline of Production or special outline, the Test Serial is considered **SATISFACTORY**.

5.6 If the validity requirements are met but the titer of the Test Serial is lower than the required minimum contained in the APHIS filed Outline of Production or special outline, the Test Serial may be retested in accordance with the 9 CFR, Part 113.8.

6. Report of test results

Record all test results on the test record. The titer of the test serial is reported as the $\log_{10}$ $TCID_{50}$ per dose.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.300, U.S. Government Printing Office, Washington, DC, 2000.

7.2 Conrath TB. Handbook of Microtiter Procedures. *Clinical and Research Applications Laboratory*, Alexandria, VA: Cooke Engineering Co, 1972.

7.3 Finney DJ. *Statistical Method in Biological Assay*, 3rd ed. London: Charles Griffin and Co, 1978.

7.4 Rose NR, Friedman H, Fahey JL, eds. Neutralization Assays. In: *Manual of Clinical Laboratory Immunology*. Washington, DC: ASM 1986.

8. Summary of revisions

This document was rewritten to meet the current NVSL/CVB QA requirements, to clarify practices currently in use in the CVB-L, and to provide additional detail. No significant changes were made from the previous protocol.

9. Appendix

Gottfried RV* (1:5):	1 ml + 1 ml Anti-Gottfried AS
	1 ml + 1 ml Anti-OSU AS
OSU RV* (1:5):	1 ml + 1 ml Anti-Gottfried AS
	1 ml + 1 ml Anti-OSU AS
Test Vaccine (1:5):	1 ml + 1 ml Anti-Gottfried AS
	1 ml + 1 ml Anti-OSU AS

* RV = Reference Virus
AS = Anti-PROTA serum